SOLID STATE OLIGONUCLEOTIDE CONSTRUCTION USING ARYLOXYCARBONYL PROTECTION AND α-EFFECT NUCLEOPHILE DEPROTECTION

This invention relates to chemical synthesis of oligonucleotides and, more particularly, this invention relates to solid phase oligonucleotide synthesis using phosphoramidite intermediates, and to construction of planar arrays of oligonucleotides.

Background

Solid phase chemical synthesis of DNA fragments is routinely performed using protected nucleoside phosphoramidites. S.L. Beaucage *et al.* (1981), *Tetrahedron Lett.* 22, 1859. In this approach, the 3'-hydroxyl of the initial (5'-protected) nucleoside is first covalently attached to the polymer support. R.C. Pless *et al.* (1975), *Nucleic Acids Res.* 2, 773 (1975). Synthesis of the oligonucleotide then proceeds by deprotection of the 5'-hydroxyl of the initial attached nucleoside, followed by coupling of this hydroxyl group with an incoming nucleoside-3'-phosphoramidite. M.D. Matteucci *et al.* (1981), *J. Am. Chem. Soc.* 103, 3185. The resulting phosphite triester is finally oxidized to a phosphorotriester to complete the internucleotide bond. R.L. Letsinger *et al.* (1976), *J. Am. Chem. Soc.* 98, 3655. The steps of deprotection, followed by coupling and then oxidation, are repeated until an oligonucleotide of the desired length and sequence is obtained.

The chemical group conventionally used for the protection of nucleoside 5'-hydroxyls is dimethoxytrityl (DMT). H.G. Khorana (1968), *Pure Appl. Chem.* 17, 349; M. Smith *et al.* (1962), *J. Am. Chem. Soc.* 84, 430. This acid labile protecting group provides a number of advantages for working with both nucleosides and oligonucleotides. The DMT group can be introduced onto a nucleoside regioselectively and in high yield. E.I. Brown *et al.* (1979), *Methods in Enzymol.* 68,109. The lipophilicity of the DMT group greatly increases the solubility of nucleosides in organic solvents. The carbocation resulting from acidic deprotection gives a strong chromophore, which can be used to indirectly monitor coupling efficiency. M.D. Matteucci *et al.* (1980), *Tetrahedron Lett.* 21, 719. And the hydrophobicity of the group can be used to aid separation on reverse-phase HPLC. C. Becker *et al.* (1985), *J. Chromatogr.* 326, 219.

The N-glycosidic linkages of oligodeoxyribonucleotides are susceptible to acid catalyzed cleavage. N.K. Kochetkov *et al.* (1972), *Organic Chemistry of Nucleic Acids* (Plenum, New York). Even when the protocol is optimized, recurrent removal of the DMT group with acid during oligonucleotide synthesis results in depurination. H. Shaller *et al.* (1963), *J. Am. Chem. Soc.* 85, 3821. The N-6-benzoyl-protected deoxyadenosine nucleotide is especially susceptible to glycosidic cleavage, resulting in reduced yield of the final oligonucleotide. J.W. Efcavitch *et al.* (1985), *Nucleosides & Nucleotides* 4, 267. There have been many attempts to address acid-catalyzed depurination utilizing alternative mixtures of acids and various solvents. E. Sonveaux (1986), *Bioorganic Chem.* 14, 274. These alternative acid mixtures have shown limited success in preventing depurination. L.J. McBride *et al.* (1986), *J. Am. Chem. Soc.* 108, 2040. More recent efforts have focused on the development of new exocyclic amine protecting groups for adenosine and guanosine: N,N-dialkylformamidine, L.J. McBride *et al.* (1983), *Tetrahedron Lett.* 24, 2953; B.C. Froehler *et al.* (1983), *Nucleic Acids Res.* 11, 8031, and N-phenoxyacetyl. J.C. Schulhof *et al.* (1987), *Nucleic Acids Res.* 15, 397. These protecting groups help stabilize the N-glycosidic linkage of purines by changing the site of initial protonation from N-1 to N-7. These have been shown to be superior to standard N-amide protection in preventing depurination. However, both the formamidine and phenoxyacetyl groups are unstable during phosphoramidite preparation and DNA synthesis.

Cleavage of the DMT group under acidic conditions gives rise to the resonance stabilized and long-lived *bis*(*p*-anisyl)phenylmethyl carbocation. P.T. Gilham *et al.* (1959), *J. Am. Chem. Soc.* 81, 4647. In an attempt to circumvent problems identified with the reversibility of DMT deprotection, pyrrole was used to quench the carbocation. C.B. Reese *et al.* (1986), *Tetrahedron Lett.* 27, 2291. The reversibility of the DMT removal necessitates using large excesses of acid to achieve quantitative deprotection. As bed volume of the polymer is increased in larger scale synthesis, a larger volume of acid is required to achieve quantitative deprotection. Operator's manual Milligen/Biosearch *8800* Nucleic Acids Synthetizer. The acid catalyzed depurination which occurs during the synthesis of oligodeoxyribonucleotides is thus increased by the scale of synthesis. M.H. Caruthers *et al.* (1982), pp. 1, in: *Genetic Engineering: Principles and Methods* J.K. Setlow *et al.*, Eds. (Plenum, New York). This makes the use of an acid labile 5'-O-protecting group less attractive for the synthesis of large quantities of oligodeoxyribonucleotides.

Considerable effort has been directed to developing 5'-O-protecting groups which can be removed under non-acidic conditions. R.L. Letsinger *et al.* (1967), *J. Am. Chem. Soc.* 89, 7147, describes using a hydrazine-labile benzoyl-propionyl group. J.F.M. deRooij *et al.*(1979), *Real. Track. Chain. Pays-Bas.* 98, 537; S. Iwai *et al.* (1988), *Tetrahedron Lett.* 29, 5383; and S. Iwai *et al.* (1988), *Nucleic Acids Res.* 16, 9443 describe using the hydrazine-labile levulinyl ester for 5'-OH protection. The cross reactivity of hydrazine with pyrimidine nucleotides as described in F. Baron *et al.* (1955), *J. Chem.* Soc. 2855 and in V. Habermann (1962), *Biochem. Biophys. Acta* 55, 999, poor selectivity of levulinic anhydride, and hydrazine cleavage of N-acyl protecting groups, as described in R.L. Letsinger *et al.* (1968), *Tetrahedron Lett.* 22, 2621, have made these two approaches impractical. H. Seliger *et al.* (1985), *Nucleosides & Nucleotides* 4, 153, describes the 5'-O-phenyl-azophenyl carbonyl (PAPco) group, which is removed by a two-step procedure involving transesterification followed by β-elimination; however, unexpectedly low and non-reproducible yields resulted. Fukuda *et al.* (1988), *Nucleic Acids Res. Symposium* Ser.19, 13, and C. Lehmann *et al.* (1989), *Nucleic Acids Res.* 17, 2389, describes application of the 9-fluorenylmethylcarbonate (Fmoc) group for 5'-protection. C. Lehmann *et al.* (1989) reports reasonable yields for the synthesis of oligonucleotides up to 20 nucleotides in length. The basic conditions required for complete deprotection of the Fmoc group, however, lead to problems with protecting group compatibility. Similarly, R.L. Letsinger *et al.* (1967), *J. Am. Chem. Soc.* 32, 296, describes using the *p*-nitrophenyloxycarbonyl group for 5'-hydroxyl protection. In all of the procedures described above utilizing base labile 5'-O-protecting groups, the requirements of high basicity and long deprotection times have severely limited their application for routine synthesis of oligonucleotides.

For *in situ* solid phase construction of planar arrays having array features presenting specific oligonucleotides, the synthesis cycle must be feature-specific. In one approach to feature-specific array construction, the entire array is serially flooded with the various reaction components through repeated synthesis cycles, and the features are selectively deprotected to permit addition of nucleosides in the desired sequence for each feature. McGall *et al.* (1997), *J. Am. Chem. Soc.* 119(22), 5082, describes employing feature-specific masking and photochemical deprotection in an adaptation of the solid phase synthesis cycle developed for porous glass surfaces (Matteucci *et al.* (1981), *supra*) to construct arrays. The yield per synthetic cycle using such photodeprotection can be too low to provide for acceptable yield of

oligonucleotides of any useful length, however (*see, e.g.*, Pirrung *et al.* (1998), *J. Org. Chem.*, 63, 241).

In another approach to feature-specific array construction, proposed in Southern (1997), *Langmuir* 13, 2833, and employed by others, the array features are defined by means of mechanical devices, or by formation of discrete droplets, each defining an address for a feature on the planar surface. In this approach, the particular phosphoramidite reagent to be added for each synthesis cycle is applied dropwise to each feature, and then the subsequent steps of the synthesis cycle (capping, oxidation, deprotection) are carried out by flooding the entire surface with the various reagents.

The use of droplet formation to constrain the specific addition of particular phosphoramidites to particular feature addresses can be problematic because of the environmental sensitivity of the reagents, and the solvent conditions necessary to achieve droplet formation. To form discrete droplets reproducibly in organic solvents, low surface energy surfaces must be employed. As the number of features in the array is increased, droplet size decreases, and the environmental sensitivity of the reagents presents more serious difficulties.

Summary of the Invention

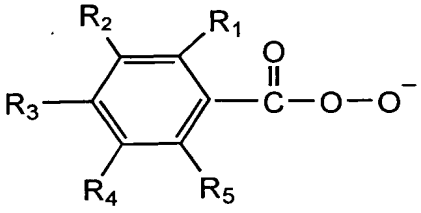
We have discovered that rapid and selective removal of suitable 5'-OH or 3'-OH protection groups following phosphoramidite condensation can be achieved by employing nucleophiles, and particularly peroxy-anions, that exhibit an α-effect under neutral or mildly basic conditions. We have further discovered that rapid and selective deprotection can be achieved under such conditions by employing carbonates, and particularly aryloxycarbonyl groups, for 5'-OH or 3'-OH protection. Deprotection of various nucleoside aryloxycarbonates, for example, using various peroxy-anions under mild pH conditions just above the pKa for the formation of the peroxy-anions results in quantitative removal of the aryloxycarbonyl group and concomitant and quantitative oxidation of the internucleotide bond. Oligonucleotides synthesized using these conditions can be isolated in high yield, and free of detectable nucleoside modifications.

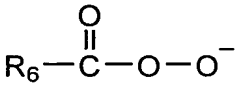
In one general aspect, the invention features a method, in an oligonucleotide synthesis, for removing a protecting group from a protected nucleoside, by reacting the protected

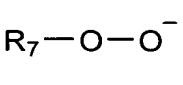
nucleoside or protected nucleotide with a nucleophile that exhibits an α-effect at conditions of mildly basic pH, and particularly at conditions of pH 10 or less.

Generally, an α-effect is an enhancement of nucleophilicity that is found when the atom adjacent a nucleophilic site bears a lone pair of electrons. As the term is used herein, a nucleophile is said to exhibit an "α-effect" if it displays a positive deviation from a Brønsted-type nucleophilicity plot. S. Hoz *et al.* (1985), *Israel J. Chem.*, 26, 313. *See also*, J.D. Aubort *et al.* (1970), *Chem. Comm.* , 1378; J.M. Brown *et al.* (1979), *J. Chem. Soc. Chem. Comm.* 171; E. Buncel *et al.*(1982), *J. Am. Chem. Soc.* 104, 4896; J.O. Edwards *et al.* (1962), *J. Amer. Chem. Soc.* 84,16; J.D. Evanseck *et al.* (1987), *J. Am. Chem Soc.* 109, 2349. The magnitude of the α-effect is dependent upon the electrophile which is paired with the specific nucleophile. J.E. McIsaac, Jr. *et al.* (1972), *J. Org. Chem.*, 37, 1037. Peroxy-anions are example of nucleophiles which exhibit strong α-effects.

Accordingly, in some embodiments the nucleophile that exhibits an α-effect is a peroxide, or a mixture of peroxides, and the pH conditions are at or above the pKa for formation of the corresponding peroxy-anion. Suitable peroxides include inorganic peroxides and organic peroxides. Suitable inorganic peroxides include those of the formula XOOH, where X is any counteranion, including for example H^+, Li^+, Na^+, K^+, Rb^+, Cs^+; and lithium peroxide or hydrogen peroxide can be particularly suitable. Suitable organic peroxides include those of the formula ROOH, where R is selected from the group consisting of alkyl, aryl, substituted alkyl and substituted aryl. More particularly, the organic peroxide will have one of the following three general structures







where R_1 - R_7 can be selected from any of the following: H, alkyl, aryl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, cycloalkenyl, alkynyl aralkynyl, cycloalkynyl, substituted aralkyl,

substituted cycloalkyl, substituted cycloalkylalkyl, substituted alkenyl, substituted cycloalkenyl, substituted alkynyl substituted aralkynyl, substituted cycloalkynyl; t-butyl-hydroperoxide or metachloroperoxybenzoic acid can be particularly suitable. We have found, as a specific example, that the *m*-chloroperbenzoic acid (MCPBA) peroxyanion exhibits a strong α-affect towards the *p*-chlorophenylcarbonate electrophile, and that, accordingly, the peroxyanion of MCPBA is a particularly effective deprotection reagent for removal of *p*-chlorophenylcarbonate protecting groups.

In another general aspect, the invention features a method for oligonucleotide synthesis, by iteratively reacting an oxycarbonyl protected nucleoside or oxycarbonyl protected nucleotide with a nucleophile that exhibits an α-effect at conditions of mildly basic pH, and particularly at conditions of pH 10 or less, and contacting the deprotected nucleoside or nucleotide with a nucleoside phosphoramidite. The internucleotide bond is oxidized following each coupling step, and in some embodiments the oxidation is effected in the iterative process by the next subsequent deprotection reaction with the α-effect nucleophile.

In some embodiments the carbonate-protected nucleoside is a nucleoside-5'-O-carbonate and the phosphoramidite nucleotide is a nucleoside-3'-phosphoramidite, and in other embodiments the carbonate-protected nucleoside is a nucleoside-3'-O-carbonate and the phosphoramidite nucleotide is a nucleoside-5'-phosphoramidite.

The method is particularly useful for solid-phase oligonucleotide synthesis, in which a protected nucleoside attached to a support surface is deprotected, then coupled with an incoming protected phosphoramidite to form a phosphate triester, which is then oxidized to a phosphorotriester to complete the internucleotide bond; and then the oligonucleotide is lengthened by iteration of the steps of deprotection, coupling, and oxidation. According to the invention, where suitable protecting groups are employed, such as oxycarbonyl groups, deprotection using a nucleophile that exhibits an α-effect at mildly basic pH concomitantly results in oxidation to form the internucleotide bond. Thus, according to the invention, oxidation to form the internucleotide bond can be carried out concomitantly with the deprotection reaction for the next subsequent coupling reaction, so that a separate oxidation step may be unnecessary.

In another general embodiment, the invention features a method for solid-phase oligonucleotide synthesis, in which a first protected nucleoside is attached to a support prior to reaction with the phosphoramidite. Particularly, a 5'-protected nucleoside is covalently attached

to the support at the 3'-hydroxyl, and is reacted with an incoming nucleoside-3'-phosphoramidite; or, a 3'-protected nucleoside is covalently attached to the support at the 5'-hydroxyl, and is reacted with an incoming nucleoside-5'-phosphoramidite.

Preferred protecting groups are those which give rise to complete and rapid deprotection when reacted with an α-effect nucleophile under mildly basic conditions (preferably at pH 10 or below).

In some embodiments the nucleoside-5'-O-carbonate or nucleoside-3'-O-carbonate is an alkyl (including substituted alkyl) or aryl (including substituted aryl) 5'-O-carbonate or 3'-O-carbonate. Suitable aryl carbonate substituents include, for example, *o*-Nitrophenylcarbonyl, *p*-Phenylazophenylcarbonyl; Phenylcarbonyl; *p*-Chlorophenylcarbonyl; and 5'-(α-methyl-2-nitropiperonyl)oxycarbonyl (MeNPOC; *see, e.g.*, McGall *et al.*, *supra*). Suitable alkyl carbonate substituents include, for example, alkyl, aralkyl, cycloalkyl, cycloalkylalkyl, alkenyl, cycloalkenyl, alkynyl aralkynyl, cycloalkynyl, substituted aralkyl, substituted cycloalkyl, substituted cycloalkylalkyl, substituted alkenyl, substituted cycloalkenyl, substituted alkynyl substituted aralkynyl, substituted cycloalkynyl; 2,2,2-Trichloro-1,1-Dimethylcarbonyl (TCBOC); and 9-Fluorenylmethylcarbonyl (FMOC) can be particularly suitable.

The invention provides for efficient solid-phase synthesis of oligonucleotides of lengths up to 25 nucleotides and greater. Treatment using an α-effect nucleophile according to the invention for removal of 5'-carbonyl protecting groups is irreversible, resulting in breakdown of the carbonate and formation of CO_2. Moreover, because such treatment results in concomitant oxidation of the internucleotide bond, the method of the invention obviates the need for a separate oxidation step.

In another general aspect, the invention features a method for making an oligonucleotide array made up of array features each presenting a specified oligonucleotide sequence at an address on an array substrate, by first treating the array substrate to protect the hydroxyl moieties on the derivatized surface from reaction with phosphoramidites, then carrying out the steps of (a) applying droplets of an α-effect nucleophile to effect a deprotection of hydroxyl moieties at selected addresses, and (b) flooding the array substrate with medium containing a selected protected phosphoramidite to permit coupling of the selected phosphoramidite onto the deprotected hydroxyl moieties at the selected addresses, and repeating the steps (a) and (b) to initiate and to sequentially build up oligonucleotides having the desired sequences at the selected addresses to complete the array features. Where the deprotected hydroxyl moiety

contacted by an incoming phosphoramidite is on an already attached nucleotide, the internucleotide bond formed between the incoming phosphoramidite and the attached nucleotide must be oxidized. In circumstances at least where the protecting group is a carbonate protecting group and the α-effect nucleophile is a peroxy-anion, this oxidation can, according to the invention, be effected concomitantly with the next subsequent deprotection step (a).

In some embodiments the oligonucleotides are synthesized in a 5' to 3' direction, so that the resulting attached nucleotides present a 3' end for ordinary enzymatic reactions; in other embodiments.

In the array construction method according to the invention, the deprotection reagents are aqueous, allowing for good droplet formation on a wide variety of array substrate surfaces. Moreover, because the selection of features employs aqueous media, small-scale discrete droplet application onto specified array addresses can be carried out by adaptation of techniques for reproducible fine droplet deposition from printing technologies.

The synthesis reaction provides irreversible deprotection resulting in evolution of CO_2, allowing for quantitative removal of protecting groups within each droplet. The phosphoramidite reactions are carried out in bulk, employing an excess of the phosphoramidite during the coupling step (b), allowing for exclusion of water by action of the excess phosphoramidite as a desiccant.

Drawings

FIG. 1 is a diagram of a synthesis cycle according to the invention, in which the oligonucleotide is constructed in a 3' to 5' direction.

FIG. 2 is a diagram of a synthesis cycle according to the invention, in which the oligonucleotide is constructed in a 5' to 3' direction.

FIGs. 3A, 3B are diagrams showing the steps in synthesis of 5'-aryloxycarbonyl-3'-nucleoside phosphoramidites and of 3'-aryloxycarbonyl-5'-nucleoside phosphoramidites, respectively.

Detailed Description

Preferred protecting groups are those which, like DMT, give rapid and selective deprotection, but which, unlike DMT, are removed under mildly basic or neutral conditions and give non-reversible reaction products. We have found that peroxy-anion deprotection of

carbonate protecting groups can provide an irreversible breakdown of the carbonate, involving formation of CO_2.

The following detailed description presents demonstrations of illustrative examples of the invention. These are presented by way of example only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Thymidine Deprotection

1. General Procedures.

The following general procedures were employed for the syntheses and the analyses. Nuclear resonance spectra (^1H, ^{13}C and ^{31}P NMR) were recorded on a Varian VXR-300 spectrometer. Tetramethylsilane was used as an internal reference for ^1H and ^{13}C NMR. An external capillary containing 85% H_3PO_4 was used as a reference for ^{31}P NMR. Downfield chemical shifts were recorded as positive values for ^{31}P NMR. Thin layer chromatography was performed on HF254 silica gel plates (Merck) in: CH_2Cl_2/MeOH, 9:1 (Solvent A), CH_2Cl_2/MeOH, 8:2 (Solvent B), ethyl acetate/ THF/Et_3N (45/45/10, v/v/v) (Solvent C). Pyridine, dichloromethane, and benzene were freshly distilled over CaH_2. Acetonitrile was distilled over P_2O_5 (solid), followed by calcium hydride, and stored over molecular sieves. Hexanes and pentanes were distilled. 5'-O-(4,4'- Dimethoxytrityl)-6-N-((di-N-butylamino)methylene)-2'-deoxyadenosine and 2-N-(di-N-butylamino)methylene-2'-deoxyguanosine were prepared according to the published procedures. Protected nucleoside derived CPG was obtained from Applied Biosystems Inc.

2. Synthesis of 5'-O-Nucleoside Carbonates.

The syntheses were conducted generally as follows. Thymidine (2 mmol) was co-evaporated with anhydrous pyridine (2 × 20 ml), then redissolved in dry pyridine (40 ml). The corresponding chloroformate (2.2 mmol) was added and the mixture stirred at room temperature (25° C) for 2 hr. The reaction was quenched with water (1 ml), then concentrated. The residual pyridine was removed by co-evaporation with toluene (40 ml).

The resulting residue was then dissolved in $CHCl_3$ (50 ml) and extracted with brine (40 ml). The aqueous layers were back-extracted with $CHCl_3$ (30 ml). The organic layers were combined, concentrated, and then loaded onto a silica gel column (100 g). The column was eluted with CH_2Cl_2 using a methanol gradient. The isolated products were evaporated to foams.

This scheme was used to synthesize a series of alkyl and aryl 5'-O-carbonates of thymidine from the corresponding chloroformates. In all cases, the best yields for the 5'-protected nucleoside were obtained when the reactions were performed at room temperature in pyridine using a slight excess of the chloroformate (1.1 eq). Under these conditions, good regioselectivity was observed with most chloroformates. However, the isolated yield of the 5'-protected nucleoside varied greatly with various alkyl or aryl substituents of the carbonate (Table I). Our attempts to increase the yield of the 5'-carbonation reactions resulted in the formation of non-regioselective products.

Table I

Isolated Yields of 5'-Protected Thymidine

with Various Alkyl and Aryl Chloroformates at Room Temperature in Pyridine

	5'-Carbonate Protected Thymidine	Isolated Yield
3a	$Cl_3C(CH_3)_2COCO_2$-dT [5'-O-TCBOC-dT]	87 %
3b	[5'-O-FMOC-dT]	90 %
3c	2-$(NO_2)C_6H_4OCO_2$-dT [5'-O-oNPh-dT]	35 %
3d	$C_6H_5N{=}NC_6H_4OCO_2$-dT [5'-O-PAP-dT]	50 %
3e	$C_6H_5OCO_2$-dT [5'-O-Ph-dT]	60 %
3f	4-$(Cl)C_6H_4OCO_2$-dT [5'-O-pClPh-dT]	60 %

The results were as follows (the various 5'-carbonate protected thymidines are numbered **3a**, **3b**, *etc.*, as in Table I).

3a. 5'-O-(2,2,2-Trichloro-1,1-Dimethylcarbonyl)Thymidine.

Yield 87%. R_F (A) = 0.40, R_F (B) = 0.70. ^{1}H NMR ($CDCl_3$+DMSO-D_6) δ: 7.33 (d, 1, H_6), 6.34 (t, J = 7 Hz, 1, $H_{1'}$), 4.45-4.08 (m, 4, $H_{3'}$, $H_{4'}$, $H_{5,5'}$,) 2.32-2.1 (m, 2, $H_{2,2'}$), 1.94-1-93 (m, 6, C-$(CH_3)_2$),1.88 (s, 3, $C^5$5-CH_3). ^{13}C NMR ($CDCl_3$+DMSO-D_6) δ: 163.27 (C-4),150.93 (0-

(CO)-O), 149.68 (C-2),134.21 (C-6), 109.71 (C-5), 104.37 C-Cl_3), 88.64 (C-Me_2), 83.39 (C-4'), 82.94 (C-1'), 62.96 (C-3'), 66.4 (C-5'), 20.02, 19.95 (C-$(CH_3)_2$), 11.6 (C^5-CH_3).

3b. 5'-O-(9-Fluorenylmethylcarbonyl)Thymidine.

Yield 90%. R_F (A) = 0.41, R_F (B) = 0.74. ^{1}H NMR ($CDCl_3$+DMSO-D_6) δ: 7.72-7.28 (m, 9, Fmoc + H_6), 6.36 (t, J = 7 Hz, 1, $H_{1'}$), 4.54-4.11 (m, 3, $CHCH_2$ (Fmoc), $H_{3'}$, $H_{4'}$, $H_{5,5'}$) 2.35-2.06 (m, 2, $H_{2,2'}$) 1.79 (s, 3, C^5-CH_3). ^{13}C NMR ($CDCl_3$+DMSO-D_6) δ: 163.87 (C-4), 154.58 (C-2), 150.28 (O-(CO)-O), 142.76, 142.71, 140.91, 127.04, 126.82, 124.59, 119.75 (Fmoc), 134.89 (C-6), 110.55 (C-5), 84.29 (C- 4'), 83.76 (C-1'), 69.47 (C-3'), 66.92 (C-5'), 46.3 (Fmoc), 39.86 (C-2'), 12.13 (C^5-CH_3).

3c. 5'-O-(*o*-Nitrophenylcarbonyl)Thymidine.

Yield 35%. R_F (A) = 0.41, R_F (B) = 0.68. ^{1}H NMR ($CDCl_3$) δ: 8.21 (d, 1, H_6), 7.89-7.53 (m, 4, aryl), 6.37 (t, J = 7 Hz, 1, $H_{1'}$), 4.53-4.17 (m, 4, $H_{3'}$, $H_{4'}$, $H_{5,5'}$), 2.33-2.03 (m, 2, $H_{2,2'}$) 1.79 (s, 3, $C^5$5-CH_3). ^{13}C NMR ($CDCl_3$) δ: 164.3 (C-4), 153.04 (O-(CO)-O), 152.21 (C-2), 144.68, 142.1, 136.5, 128.36, 126.65, 125.68 (C_6H_4), 136.33 (C-6), 111.05 (C-5), 85.44 (C- 1'), 84.62 (C-4'), 71.54 (C-5'), 69.67 (C-3'), 40.15 (C-2'), 12.4 (C_5-CH_3).

3d. 5'-O-(*p*-Phenylazophenylcarbonyl)Thymidine.

Yield 50%. R_F (A) = 0.41, R_F (B) = 0.75. ^{1}H NMR ($CDCl_3$) δ: 7.94-7.28 (m, 10, $H_6$6+aryl(PAP)), 6.31 (t, J = 7 Hz, 1, $H_{1'}$), 4.5-4.12 (a, 4, $H_{3'}$, $H_{4'}$, $H_{5,5'}$), 2.33 - 2.19 (m, 2, $H_{2,2'}$), 1.86 (s, 3, C_5-CH_3). ^{13}C NMR ($CDCl_3$) δ: 164.44 (C-4), 152.33 (O-(CO)-O), 152.1 (C-2), 152.86, 152.16, 150.55, 150.23, 131.05, 128.84, 123.86, 122.54, 121.21 (PAP), 135.56 (C-6), 110.92 (C-5), 84.65 (C-1'), 83.55 (C-4'), 70.13 (C-5'), 67-53 (C-3'), 39.73 (C-2'), 11.93 (C^5-CH_3).

3e. 5'-O-(Phenylcarbonyl)Thymidine.

Yield 60%. R_F (A) = 0.41, R_F (B) = 0.71. ^{1}H NMR ($CDCl_3$) δ: 7.54-7.19 (m, 6, H_6+aryl), 6.34 (t, J = 7 Hz, 1, $H_{1'}$) 4.52-4.12 (m, 4, $H_{3'}$, $H_{4'}$, $H_{5,5'}$), 2.3-2 (a, 2, $H_{2,2'}$), 1.78 (s, 3, C^5-CH_3). ^{13}C NMR (DMSOd-$_6$+$(CD_3)_2CO$) δ: 164.36 (C-4), 152.21 (O-(CO)-O), 151.35 (C-2), 154.2, 130.42, 126.97, 121.99 (C_6H_4), 136.61 (C-6), 111.11 (C-5), 85.44 (C-1'), 84.84 (C-4'), 71.73 (C-5'), 68.83 (C-3'), 40.21 (C-2'),12.5 (C^5-CH_3).

3f. 5'-O-(*p*-Chlorophenylcarbonyl)Thymidine.

Yield 60%. R_F (A) = 0.42, R_F (B) = 0.73. ^{1}H NMR ($CDCl_3$) δ: 7.9 (d, 1, H_6), 7.44-7.16 (m, 5, aryl), 6.34 (t, J = 7 Hz, 1, $H_{1'}$), 4.6-4.12 (m, 4, $H_{3'}$, $H_{4'}$, $H_{5,5'}$). 2.3-2.05 (m, 2, $H_{2,2'}$), 1.74 (s, 3, C^5-CH_3). ^{13}C NMR ($CDCl_3$) δ: 164.4 (C-4), 153.23 (O-(CO)-O), 151.4 (C-2), 149.39, 139.86,

129.73, 122.23 (C_6H_4), 136.6 (C-6), 111.1 (C-5), 85.41 (C-1'), 84.8 (C-4'), 71.52 (C-3'), 67.53 (C-5'), 40.25 (C-2'), 12.49 (C^5CH_3).

3. Synthesis of 5'-O-DMT-3'-O-R-Thymidine.

The 3'-hydroxyl group of 5'-O-DMT-thymidine was protected with a phenyloxycarbonyl (referred to as 4a below), a benzoyl (4b), and an acetyl (4c) group, as follows. 5'-O-(4,4'-Dimethoxytrityl)-thymidine (1 mmol) was co-evaporated 3 times with anhydrous pyridine, then redissolved in 20 ml of pyridine. Corresponding chloroformates (1.1 mmol) were added to the nucleoside mixture. After stirring for 6 hr, the reaction was quenched with water (100 ml) and concentrated. Residues of pyridine were removed by co-evaporation with toluene (2 × 20 ml).

The resulting gum was dissolved in CH_2Cl_2, extracted with 10% aqueous $NaHCO_3$, and dried over Na_2SO_4. After concentration, the product was loaded onto a silica gel column (50 g) and eluted with CH_2Cl_2 using a methanol gradient (0-3%). Product fractions were collected and concentrated to a foam.

The results were as follows.

4a. 5'-O-(4,4'-Dimethoxytrityl)-3'-O-Phenylcarbonyl Thymidine.

Yield 80%. R_F (A) = 0.74, R_F (B) = 0.91. ^1H NMR ($CDCl_3$) δ: 7.65-6.83 (m, 18, H_6+DMTr+aryl), 6.57 (t, J = 7 Hz, 1, $H_{1'}$), 5.45 (m, 1, $H_{3'}$) 4.34 (m, 1, $H_{4'}$), 3.79 (s, 6, OCH_3), 3.54 (m, 2, $H_{5,5'}$), 2.72-2.52 (m, 2, $H_{2,2'}$), 1.41 (s, 3, C^5-CH3).

4b. 5'-O-(4,4'-Dimethoxytrityl)-3'-O-Benzoylthymidine.

Yield 90%. R_F (A) = 0.72, R_F (B) = 0.91. ^1H NMR ($CDCl_3$) δ: 8.07-6.85 (m, 18, H_6+DMTr+aryl), 6.58 (t, J = 7 Hz, 1, $H_{1'}$), 5.45 (m, 1, $H_{3'}$), 4.14 (m, 1, $H_{4'}$), 3.79 (s, 6, OCH_3), 3.57 (m, 2, $H_{5,5'}$) 2.63 (m, 2, $H_{2,2'}$), 1.42 (s, 3, C^5-CH_3).

4c. 5'-O-(4,4'-Dimethoxytrityl)-3'-O-Acetylthymidine.

Yield 90%. R_F (A) = 0.67, R_F (B) = 0.89. ^1H NMR ($CDCl_3$) δ: 7.62 (s, 1, H_6),7.4-6.82 (m, 13, DMTr), 6.46 (t, J = 7 Hz, 1, $H_{1'}$), 5.45 (m, 1, $H_{3'}$), 4.14 (m, 1, $H_{4'}$), 3.78 (s, 6, OCH_3), 3.47 (m, 2, $H_{5,5'}$), 2.45 (m, 2, $H_{2,2'}$) 2.08 (s, 3, CO-CH_3), 1.39 (s, 3, C^5-CH_3).

4. Nucleoside deprotection by peroxy-anions.

Deprotection reactions were carried out using peroxy-anions on alkyl and aryl 5'-O-carbonates of thymidine synthesized as described above. The reactions were monitored by TLC for complete conversion of the starting material to thymidine. A wide variety of peroxy-anions, known to exhibit strong α-effects, were screened for their ability to cleave 5'-O-carbonates of thymidine. Peroxy-anion solutions active in cleavage of the 5'-O-carbonates were buffered at a

variety of pH conditions. The cleavage activity of these peroxy-anion solutions was shown to be rapid only at pH conditions above the pKa for the formation of the anion. Peroxy-anion solutions which gave rapid deprotection at mildly basic pH are identified as **i, ii, iii, iv** along with specific pH conditions in Table II. The ability of these peroxy-anion solutions (**i - iv**) to completely deprotect the 5'-O-carbonates of thymidine (**3a - 3f**) is summarized in Table III.

5. Selectivity of Various Peroxy-Anion Solutions for Deprotection of Carbonates.

The 3'-hydroxyl group of 5'-O-DMT-thymidine was protected with a phenyloxycarbonyl (4a), a benzoyl (4b), and an acetyl (4c) group. The stability of these 3'-protecting groups was determined by TLC using deprotection conditions **iii** and **iv** (Table II). Under both these conditions, the phenyl-carbonate was completely removed in less than 2 min. The 3'-benzoyl group was completely stable under both conditions for 140 min. The 3'-acetyl group was cleaved to a small extent (less than 3%) over the 140 min exposure to deprotection condition **iii** (pH 10.0). The 3-benzoyl group was completely stable for the 140 min exposure to condition **iv**.

6. Selectivity of Deprotection on Solid-Support Attached Nucleosides.

The demonstration of stability at the 3' position was then extended to the succinate linker commonly used for the attachment of nucleosides to Control Pore Glass, as follows. 5'-DMT-thymidine attached to Long Chain Alkyl Amine Control Pore Glass (LCAA/CPG) through a 3'-succinate linkage was obtained from a commercial source. This solid-support attached nucleoside was then exposed to deprotection conditions **i - iv**. The stability of the 3'-linkage was determined spectrophotometrically based upon the evolution of the trityl cation during subsequent treatment with toluene sulfonic acid in anhydrous acetonitrile. Deprotection conditions **i** and **ii** gave complete cleavage of the 3'-succinate in 20 min. Deprotection conditions **iii** and **iv** gave less than 2% cleavage of the 3'-succinate after 20 hrs.

Table II

Peroxy-Anion Solutions Shown to be Active in Deprotection

of 5'-Carbonates of Thymidine

i		ii		iii		iv		v	
LiOH	0.8 M	LiOH	0.8 M	LiOH	0.8 M	MCPBA	0.065 M	tBuOOH	0.1 M
H_2O_2	1.1 M	H_2O_2	1.1 M	H_2O_2	1.1 M	H_2O_2	1.1 M	H_2O_2	1.1 M
Tris	15 ml	Tris	15 ml	Tris	15 ml	Tris	15 ml	Tris	15 ml
H_2O	10 ml	H_2O	10 ml	H_2O	10 ml	H_2O	10 ml	H_2O	10 ml
Dioxane	50 ml	Dioxane	50 ml	Dioxane	50 ml	Dioxane	50 ml	Dioxane	50 ml
				MCPBA	0.065 M				
(pH 12.0)		(pH 12.0)		(pH 10.0)		(pH 9.0)		(pH 9.0)	

Tris = *tris*(hydroxymethylamino)-methane, 2.5 M (Tris-Base)

MCPBA = metachloroperoxybenzoic acid

tBuOOH = t-butyl-hydroperoxide

Table III

Times Required for Complete Conversion

of Protected Nucleosides **3a**, **3b**, **3c**, **3d**, **3e**, **3f** (Table I)

Using Peroxy-Anion Solutions **i**, **ii**, **iii**, **iv**, **v** (Table II)

5'-Carbonate- dT Compounds	Reaction Completion Times for Deprotection Solutions				
	i	ii	iii	iv	v
3a	< 1 min	< 1 min	< 12 min	-	-
3b	> 1 hr	< 1 min	> 3 hr	-	-
3c	< 1 min	< 1 min	< 1 min	-	-
3d	< 1 min	< 1 min	< 1 min	< 1 min	> 12 hr
3e	< 1 min	< 1 min	< 1 min	< 2 min	< 12 hr
3f	< 1 min	< 1 min	< 1 min	< 1 min	< 12 hr

EXAMPLE 2

Oligothymidylate Deprotection and Internucleotide Bond Oxidation by Peroxy-Anions

1. Oligonucleotide Synthesis on Controlled Pore Glass.

Oligonucleotides were synthesized on CPG using an automated DNA synthesizer (ABI model 380A). The synthesis cycle used for 5'-DMT protected nucleosidephosphoramidites (Cycle 1) is shown in Table VI. This cycle was initially modified for the use of 5'-carbonate protected nucleosidephosphoramidites simply by substituting the alternative deprotection mixtures for the 3% TCA solution (Step 8, Table VI) and varying the exposure times. For the synthesis of longer sequences using 5-carbonate protected nucleosidephosphoramidites, it was necessary to separate the deprotection mixture into a two-component system (Table V). The separation of the deprotection mixture was accomplished using the capping ports on the synthesizer, and thus necessitated elimination of the capping step from the synthesis cycle. The optimized cycle for synthesis using 5'-carbonate protected nucleosidephosphoramidites (Cycle 2) is shown in Table VI.

Table V

Two-Component System for Storage of Deprotection Solution **iii** (Table II)

Solution **A**	30 % H_2O_2 (10 ml), LiOH (280 mg), dioxane (7.5 ml), 2.5 M Tris-Base (15 ml), water (42.5 ml)
Solution **B**	50 - 60 % MCPBA (1.78 g), dioxane (42.5 ml)

Table VI

Oligonucleotide Synthesis Cycles

Step #	Function	Reagent	Cycle 1 Time, sec.	Cycle 2 Time, sec.
1	Wash	Acetonitrile	25	25
2	Coupling	Amidite (0.15 M, 30 eq) Tetrazole (0.5 M, 120 eq) in Anhydrous Acetonitrile	2 × 30	2 × 30
3	Wash	Acetonitrile	5	5
4	Capping	N-Methylmidazole/2,6-Lutidine/Acetic Anhydride/THF (1/1/1/2, vol/vol/vol/vol)	40	-

5	Oxidation	0.1 M I_2 in THF/Lutidine/Water (80/40/2, vol/vol/vol)	30	-
6	Wash	Acetonitrile	25	-
7	Wash	Dichloromethane (Cycle 1) 1,4-Dioxane (Cycle 2)	25	 25
8	Deblock	3 % TCA in CH_2Cl_2 (Cycle 1) 1:1 mix of Solution **A** & Solution **B** from Table V (Cycle 2)	2 × 30	 480
9	Wash	Dichloromethane (Cycle 1) 1,4-Dioxane (Cycle 2)	25	 25

2. Analysis of Oligonucleotides by HPLC.

The oligonucleotides synthesized on the solid support were deprotected with concentrated ammonium hydroxide (55° C, 24 hr). The ammonium hydroxide solutions were removed from the support and evaporated to dryness. The crude oligonucleotides were reconstituted in distilled water and stored at -20° C.

HPLC analysis was performed by ion-exchange HPLC (Nucleogen 60-7DEAE, 4 mm ID x 125 mm). Oligonucleotides were eluted from the column with a LiCl gradient (0.0-0.7 M) in a water/acetonitrile (60/40, v/v) buffer containing sodium acetate (0.002 M, pH 6.0).

3. Solid-Support Deprotection of 5'-O-Carbonates of Thymidine.

The deprotection efficiency of peroxy-anion solutions on oligonucleotides was determined by the synthesis of oligothymidylate tetramers. The 5'-O-arylcarbonates of thymidine (*see*, Table 1: 3c, 3d, 3e, 3f) were converted to the corresponding 3'-O-(2-cyanoethyl)- N,N-diisopropylphosphoramidite by procedures described generally in A.D. Barone *et al.* (1984), *Nucleic Acids Res.* 12, 4051, as follows.

Synthesis of the 2-cyanoethyl-N,N,N',N'-tetraisopropyl-phosphorodiamidite phosphine was performed according the procedure described in A. Kraszewski *et al.* (1987), *Nucleic Acids Res.* 18, 177. The resulting product was purified by distillation from CsF. The product was obtained in 60% yield. Purity was confirmed by ^{31}P NMR ($CDCl_3$) δ: 123.8 ppm.

Thymidyl-3'-5'-thymidine was synthesized on solid-support using 5'-O-dimethoxytrityl-3'-O-(2-cyanoethyl)-N,N-diisopropylaminothymidinephosphoramidite. The dimer was elongated to a trimer using a 5'-O-aryloxycarbonyl-3'-O-(2-cyanoethyl)-N,N-diisopropylaminothymidinephosphoramidite and synthesis cycle 1 (Table VI). Deprotection of

the carbonate was then attempted using deprotection mixture **iii**, at 1 min increments, from 1-15 min. The extent of deprotection was determined by the yield of the subsequent coupling reaction using a standard 5'-DMT-dT phosphoramidite. Deprotection efficiency for the 5'-O-arylcarbonate was determined using ion-exchange HPLC. The percent deprotection was calculated by integration and normalization of peak areas for the corresponding trimers and tetramers, assuming quantitative coupling reactions. The optimum deprotection time and extent of deprotection for each aryloxycarbonyl group is summarized in Table IV.

Table IV

Optimum Deprotection Times Determined

for 5'-Arylcarbonates of Thymidine on Control Pore Glass

Using Deprotection Solution **iii** (Table II)

5'-Carbonate dT Compounds	Optimum Deprotection Time	Deprotection Efficiency
3c	5 min	80 %
3d	1 min	94 %
3e	7 min	98 %
3f	3 min]	98 %

4. Solid Support Synthesis and Internucleotide Bond Oxidation.

Several oligothymidylate tetramers were synthesized on Control Pore Glass using 5'-O-*p*-chlorophenyloxycarbonyl-3'-O-(2-cyanoethyl)-N,N-diisopropylaminothymidinephosphoramidite. These syntheses were performed on a 1 μmol scale using an automated DNA synthesizer. The only modification from the standard 1 μmol synthesis cycle (Cycle 1, Table VI) was the use of deprotection mixture **iii** (7 min) in place of 3% TCA in dichloromethane. The resulting tetramers were compared to oligothymidylate tetramers synthesized using the standard DMT protected phosphoramidites of thymidine. These tetramers were then analyzed using ion-exchange HPLC. There were no detectable differences in the yield or purity of any of the oligomers.

Oligothymidylate tetramers were then synthesized using this same synthesis cycle, which was again modified by the removal of the iodine oxidation step. This concomitant deprotection and oxidation cycle produced tetramers of identical yield and purity to the

standard DMT phosphoramidite synthesis. Decomposition of MCPBA in the presence of LiOH results in the deprotection mixture being effective for only a few hours. In order to synthesize longer sequences, it was necessary to separate the deprotection mixture into a two component system (Table V). This was accomplished using the capping ports on the automated DNA synthesizer. Separating the LiOH from the MCPBA and mixing just prior to deprotection allows the reagents to remain effective for several days. Oligonucleotide synthesis using 5'-O-arylcarbonate nucleoside phosphoramidites was carried out with and without acetic anhydride capping. No adverse effects on the yield of final product or increases in the appearance of n-1 products were observed in absence of capping. This is contrary to what is seen with the use of DMT protected phosphoramidites in the absence of capping. Anion-exchange HPLC profiles of crude synthesis products of oligothymidylate decamers were produced. Product purity and yield of full-length oligonucleotides, using peroxyanion deprotection of 5'-O-carbonates in absence of acetic anhydride capping and iodine oxidation (Cycle 2, Table VI), were comparable to or better than those obtained using DMT phosphoramidites and the standard synthesis cycle.

EXAMPLE 3

No Oxidation During Peroxy-Anion Deprotection of 5'-O-DMT-Protected Cytosine, Adenine, Uracil, Thymidine or Guanosine Nucleosides

The unprotected heterocyclic bases cytosine and adenine are susceptible to N-oxidation by peracids and peroxides under stringent conditions; and oxidative reactions which result in ring cleavage of uracil, thymidine and guanosine in the presence of highly concentrated peroxides at elevated temperatures have been described. 5-O-DMT-protected nucleosides, N-protected with a (di-N-butylamino)methylene group, were dissolved in deprotection mixture **iii** and allowed to react for 24 hrs. The tritylated nucleosides were extracted from the aqueous deprotection mixture with $CHCl_3$ and analyzed by ^{13}C NMR and TLC. Neither formation of N-oxides nor attack at the 5,6-double bond of thymidine (leading to ring cleavage) was detected.

EXAMPLE 4

Synthesis of Mixed Oligonucleotides

This example demonstrates extension of the method of the invention to synthesis of mixed oligonucleotide sequences, employing substituted aryl carbonate protected

phosphoramidite synthons, and following each coupling reaction by treatment with a mixture of peroxy-anions at mild pH (less than 10) to deprotect and concomitantly oxidize the internucleotide linkage.

The method is high-yielding, and effective for the four main 2'-deoxynucleotides. Synthesis in both the 3' - 5' direction and the 5' - 3' direction were carried out, with equal effect.

1. Protected Phosphoramidite Synthesis.

Generally, the protected nucleoside phosphoramidites were prepared as follows. The 3'- or 5'-protected nucleoside (5.00 mmol) and tetrazole (175 mg, 2.50 mmol) were dried under vacuum for 24h and then dissolved in trichloromethane (100 mL). 2-cyanoethyl-*N,N,N',N'*-tetraisopropyl phosphane (2.06 mL, 6.50 mmol) was added in one portion and the mixture stirred over 1 hour. The reaction mixture was washed with sat. $NaHCO_3$ (150 mL) and brine (150 mL), dried over $MgSO_4$ and applied directly to the top of a silica column equilibrated with hexanes. The dichloromethane was flashed off the column with hexanes, and the product eluted as a mixture of diastereoisomers using 1/1 hexanes/ethyl acetate then ethyl acetate. After evaporation of solvents *in vacuo* and coevaporation with dichloromethane, products were isolated as friable, white, glassy solids in yields varying from 70% to 90%.

The four 5'-aryloxycarbonyl-3'-nucleoside phosphoramidites were prepared by the straightforward two-step procedure shown generally in FIG. 3A. In a first step, commercially available base protected 2'-oligodeoxynucleosides were selectively aryl carbonate protected at the 5' position by treatment with 4-chlorophenyl chloroformate in dilute anhydrous pyridine to yield 5'-aryloxycarbonyl protected compounds in moderate to good yield. The use of more concentrated reaction mixtures resulted in an increase in the amounts of isolated 3'- and 3', 5'-*bis*-aryloxycarbonyl-protected materials. In a second step, the resulting compounds were phosphitylated using the method described in Barone *et al.*, *supra*, to furnish high yields following column chromatography.

Synthesis of the four 3'-aryloxycarbonyl-5'-nucleoside phosphoramidites were prepared by the three-step procedure shown in FIG 3B.

2. Deprotection Mixture.

The deprotection mixture was formulated in two parts, which were mixed immediately prior to use. Solution A: 3.1% w/v lithium hydroxide monohydrate (10 mL), 1.5 M 2-amino-2-methyl-1-propanol pH 10.3 (15 mL), 1,4 dioxane (17.5 mL). Solution B: 1,4-dioxane (32.5 mL), 50 - 83% 3-chloroperbenzoic acid (1.78 g), 30% hydrogen peroxide (12 mL). The initial

pH of the deprotection mixture was 9.6 ± 0.05. For pH dependence studies, the initial deprotection mixture was altered by varying the strength of the lithium hydroxide solution.

3. Synthesis of Mixed-Sequence Oligonucleotides.

A series of model oligodinucleotides was synthesized, having sequences 3'-$T_3AT_2AT_3$-5', 3'-$T_3CT_2CT_3$-5', 3'-$T_3GT_2GT_3$-5', 3'-TACGT-5', 3'TACGTACGT-5', 3'-TA_7T-5', 5'-TACGT-3', and 5'-TACGTACGT-3'. RP HPLC traces of the resulting products confirmed the results.

4. Stability of Base Protecting Groups in the Deprotection Mixture.

The stability of the standard base protecting groups A^{Bz}, C^{Bz}, and G^{ibu} during exposure to the deprotection mixture was tested by incubating 5'-DMT base-protected deoxynucleosides at room temperature with a large excess of the deprotection mixture. The extent of cleavage of the base protecting groups over time was measured by TLC. The approximate $T_{1/2}$ values for A^{Bz}, C^{Bz}, and G^{ibu} were approximately ½ hour, 2 hours, and 1 day, respectively, and unlikely to present difficulties for syntheses.

EXAMPLE 5

Array Synthesis

This Example presents application of the method of the invention to construction of oligonucleotide arrays. As demonstrated in the foregoing account, solid phase synthesis of oligonucleotides can be effectively carried out according to the invention in either the 5' - 3' direction or the 3' - 5' direction, yielding oligonucleotides of specific sequence having lengths up to 25 nucleotides and longer.

The method can be particularly useful for *in situ* phosphoramidite-based construction of oligonucleotide arrays. Particularly, the method provides for precision chemical deprotection of selected sites on an array substrate surface; because the deprotection mixture is in an aqueous medium, printing techniques for precision fine-scale deposition of inks can be adapted for precise deposition of fine discrete droplets of the deprotection mixture onto the array surface, effecting precisely located site-specific removal of hydroxyl protecting groups either on the hydroxyl derivatized substrate surface itself (to initiate oligonucleotide synthesis) or on already attached nucleotides. This deprotection step can be followed by flooding the surface with a selected protected nucleoside phosphoramidite, to effect coupling at the deprotected hydroxyl moieties. Oxidation of the internucleotide bond completes the addition process; According to the invention, the use of α-effect nucleophiles as deprotection agents has the concomitant effect

of oxidizing the internucleotide bond. Accordingly, the oxidation step for attached nucleotides can be carried out concomitantly with the deprotection as the next subsequent deprotection step is carried out by droplet application at that feature address.

The surface can be constructed of any material that can be derivatized to permit attachment of the first nucleoside; examples of suitable materials include any of a variety of glasses or other silica-based materials (such as, for example, Control Pore Glass), and plastics. Derivatization of the surface can be carried out using any of a variety of techniques for solid-phase synthetic processes, and many of these are well known.

These examples demonstrate effective rapid and selective deprotection of 5'- and of 3'-protected nucleosides by use of α-effect nucleophile deprotection reagents under mildly basic conditions. The use of peroxy-anion deprotection of nucleotide 5'-carbonates can provide a useful tool in the synthesis of oligonucleotides and modified oligonucleotides. Peroxy-anion deprotection of carbonates may also become a useful tool for the rapid deprotection of other carbonate protecting groups under mild pH conditions.

Particularly, for example, removal of aryloxycarbonyl groups by peroxy-anions was shown to occur rapidly and under mild pH conditions (pH 10 or lower). Carbonate deprotection is followed by the formation of the phenol derivative and carbon dioxide. Loss of CO_2 assures irreversibility of the deprotection.

Electrophile specificity for peroxy-anion mixtures was demonstrated for the deprotection of carbonates in the presence of acetyl, benzoyl, and succinyl esters. Carbonate deprotection reactions were also shown to be rapid and efficient on solid support. The synthesis of oligothymidilates on Control Pore Glass, utilizing 5'-aryloxycarbonyl protected nucleoside phosphoramidites and peroxy-anion deprotection, gave products of comparable yield and purity to those synthesized with commonly used acid labile DMT protecting groups.

Using peroxy-anion deprotection, iodine oxidation was omitted without adverse effects. This allows deprotection and oxidation to be performed as a single step using a single reagent. The peroxy-anion reagent, chosen for its optimum deprotection characteristics, can be stored for several days as a two component system and can be easily integrated into a synthesis cycle on a standard DNA synthesizer.

The use of the dilute oxidants under mild conditions according to the invention gives effective deprotection without detectable modification of the heterocyclic residues, and specifically without formation of N-oxides and without oxidative ring cleavage.

Other embodiments, as will appear to the skilled artisan. are contemplated by the invention and are within the scope of the claims.